Evaluation of 'white-spotted kidneys' associated with leptospirosis by polymerase chain reaction based *LipL32* gene in slaughtered cows

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The presence of white spots in the kidneys of cattle at slaughter (so-called white-spotted kidneys) can be an indication of infection with *Leptospira*, a spirochaete of public health concern because it causes zoonotic disease. In this study, 24 kidneys of 180 slaughtered cows (13.3%) showed focal to multifocal white spots at inspection. These kidneys, together with matching urine (n = 18) and blood (n = 24) samples, were examined by polymerase chain reaction (PCR) targeting the *LipL32* gene. Leptospiral deoxyribonucleic acid (DNA) was detected in 19 (79.2%) out of 24 kidneys, as well as 7 (29.2%) blood and 10 (55.5%) urine samples of cows with white spots in their kidneys. Histopathological findings revealed multifocal infiltration of mononuclear cells, including lymphocytes and a few plasma cells in the renal interstitial tissues. In addition, 14 apparently normal kidneys and associated urine and blood samples were similarly examined by PCR but did not provide any positive results. In this study, high detection of leptospirosis in kidneys with interstitial nephritis suggests that *Leptospira* spp. are associated with white spotted kidneys. The present findings indicate that white spotted kidneys can be due to leptospirosis in this region in southwestern Iran, which indicates an increased risk of zoonotic disease. The data show that *LipL32*-based primers are useful for PCR-based diagnosis of leptospirosis.

Introduction

Leptospirosis is an acute febrile and septicaemic disease caused by spirochaetes of the family *Leptospiraceae*, which includes saprophytic and pathogenic bacteria. Pathogenic leptospires are important in public health because they cause zoonotic disease (Bharti *et al*. 2003; Hernández-Rodríguez *et al*. 2011; Levett 2001; McBride *et al*. 2005). A wide range of host species, including humans, wildlife including rodents and carnivores, and domestic animals, act as reservoirs for *Leptospira* (Jorge *et al*. 2011; Liu *et al*. 2006). Humans may be affected after direct contact with infected urine or more often indirectly via exposure to water or soil contaminated by the urine of infected animals (Tansuphasiri *et al*. 2006; Vijayachari, Sugunan & Shriram 2008).

After leptospiraemia in the host, the kidneys are the main tissue for localisation of the organism and the chronic lesion of the disease is focal chronic interstitial nephritis (FCIN) (Yang, Wu & Pan 2001), known as white-spotted kidneys. This lesion is a common finding in clinically healthy cattle at slaughter (Maxie 1993; Uzal *et al*. 2002). Although several pathogens can cause white spots in kidneys, FCIN is frequently attributed to current or previous leptospiral infection by veterinarians in the abattoir (Uzal *et al*. 2002). Clinical signs and necropsy findings of leptospirosis are not pathognomonic and may be mistaken for those produced by other pathogens. Therefore, clinical diagnosis alone is not sufficient and must be accompanied by complementary tests in order to achieve precise diagnostic results and design proper disease control strategies (Agudelo-Florez, Restrepo & Lotero 2006; Gumussoy *et al*. 2009).

In domestic animals such as cattle, leptospirosis causes septicaemia, icterus, anaemia and haemoglobinuria and is responsible for serious economic losses, especially in the meat and dairy industry, due to abortion, mastitis and a decline in milk production (Quinn *et al*. 2002). The kidney and genital organs are the main target tissues in infected cattle. Infected cattle may not show any clinical signs of disease, but excrete the organisms in their urine. Cattle therefore play an important role in spreading the infection to other susceptible animals and to human populations at risk, such as farmers and veterinarians (Levett 2001; Mineiro *et al*. 2011). The aim of this study was to determine the correlation between white-spotted kidneys with leptospirosis in cattle at an abattoir, using the polymerase chain reaction (PCR) technique. The urine and blood samples of the cattle with lesions in the kidneys were also examined for evidence of *Leptospira* spp.

Materials and methods

Study area

Chaharmahalva Bakhtiary province is an historic and beautiful area in southwestern Iran. The province has an area of 16 533 km^2, situated at the centre of the Zagros mountains, between latitudes 31°, 4′ S and 42°, 4′ N and longitudes 49°, 39′ W and 51°, 21′ E. The rainfall in the province is derived mostly from Mediterranean and Sudanese atmospheric flows from the west and south that affect the region for 8 months (from October to May). The weather in winter is rather cold and minimum temperatures may reach −20 °C. The average rainfall of the province is about 560 mm.

Sample collection

The kidneys of 180 slaughtered cows 1–4 years old were grossly examined for white-spotted kidneys from December 2010 to March 2011 at Shahrekord abattoir. The animals were slaughtered for human consumption. The kidneys of 24 cows (13.3%) showed macroscopic, focal to multifocal white spots at inspection. Samples of these kidneys were taken for pathological and PCR investigations. At the same time, urine samples were collected directly during slaughter from the urinary bladder with a sterile needle. Before slaughter, blood was collected into tubes containing anticoagulant and correlated with the carcass number in the slaughter line. To obtain the buffy coat, blood samples were centrifuged at 10000 x g for 10 minutes and the buffy coat was kept in 1.5 mL microtubes. Subsequently, all kidney, urine and buffy coat samples were stored at −20 °C until examination. In addition, samples of kidney, urine and blood were similarly taken from 14 cattle with no gross lesions in their kidneys as a control group.

Polymerase chain reaction

Deoxyribonucleic acid (DNA) was extracted from frozen kidneys, buffy coat and urine samples with a high yield DNA purification kit (Cinnagen Inc, PN811SC, Iran), according to the manufacturer's instructions. Polymerase chain reaction based on the *LipL32* gene was performed using the primers 5′ATCTCCGTTGCACTCTTTGC3′, 5′ACCATCATCATCATCGTCCA3′ as previously described by Tansuphasiri *et al*. (2006). This set of primers was designed to distinguish between pathogenic and saprophytic *Leptospira* species, because the *LipL32* gene is amplified only in pathogenic species (Tansuphasiri *et al.* 2006).

Polymerase chain reaction amplification was performed using the following programme: an initial cycle of denaturation at 94 °C for 3 min, 30 cycles of denaturation at 94 °C for 1 min, annealing at 60 °C for 90 s, extension at 72 °C for 20 min, and a final extension at 72 °C for 10 min and holding at 4 °C. The amplified products were analysed by electrophoresis on ethidium bromide-stained 2% agarose gels and the results were observed using Ultraviolet (UV) light. A sample was considered positive when the 474 bp DNA band was obtained (Vital-Brazil *et al*. 2010).

Histopathological investigation

Tissue samples of kidneys 1 cm^3 thick were fixed in 10% neutral buffered formalin for histopathological examination. The samples were then dehydrated in graded ethanol and embedded in paraffin. Sections 5 µm thick were stained with haematoxylin and eosin and examined with an ordinary light microscope.

Ethical considerations

The study was approved by the local ethics committee of our faculty, in accordance with the ethics standards of 'Principles of Laboratory Animal Care'.

Results

Polymerase Chain Reaction

In the present study, the results of a PCR method targeting the *LipL32* gene on DNA extracted from kidneys with white spots, urine and buffy coat samples are shown in Table 1. Leptospiral DNA was detected in 19 out of 24 kidneys (79.2%) with focal to multifocal white spots, whilst most animals were serologically negative for *Leptospira* spp. by the PCR technique. Only 7 of 24 buffy coat samples (29.2%) were positive by PCR. Out of 18 urine samples from the cows with white spots in their kidneys, 10 (55.5%) were PCR positive (Figure 1). Amplicons of the expected size were not detected in kidney, urine or buffy coat samples from 14 cows with apparently normal kidneys.

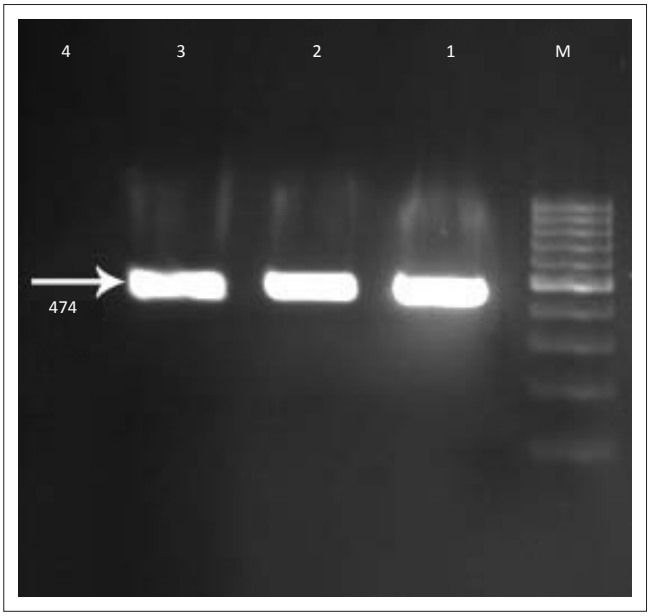
Gross and histopathological findings

Grossly, the kidneys examined showed pale, focal to multifocal spots between 1 mm and 5 mm diameter that were randomly distributed on the surface of kidneys and demarcated from adjacent tissues. On cut section, these spots formed pale wedges in the cortex with their bases under the renal capsule. Histopathologically, focal aggregation of mononuclear cells especially lymphocytes and a few plasma cells were visible in the renal interstitial tissue. The cortex was

TABLE 1: Detection of leptospiral deoxyribonucleic acid using *LipL32*-based polymerase chain reaction in blood, urine and kidney tissues of cattle with normal kidney and white-spotted kidneys.

Experimental design	White spotted kidney			Normal kidney		
	Kidneys	Blood	Urine	Kidneys	Blood	Urine
Inspected carcasses (180)	24/180	24/180	18/180	14	14	14
LipL32-based PCR positive	19/24	7/24	10/24	0	0	0
Prevalence (%)†	79.2	29.2	55.5	0	0	0

PCR, polymerase chain reaction.
†, Suspected kidneys = 13.3%.



M, 100 bp molecular weight markers; lane 1-3, positive amplification (474 bp); lane 4, negative amplification.

FIGURE 1: Detection of *Leptospira* deoxyribonucleic acid in kidney tissue of a cow by polymerase chain reaction-based *LipL32* gene.

the area most affected, followed by the cortico-medullary junction. Mononuclear cells were also infiltrated around the glomeruli.

Trustworthiness

The investigators who undertook the gross observations, histopathological and molecular studies and analysis in the present study were unaware of the experimental design and grouping details. The histopathological studies were blindly undertaken by three different pathologists.

Discussion

More than 240 serovars of leptospires are recognised, comprising 23 serogroups (Collins 2006). The clinical signs of the disease are nonspecific and it is frequently lethal in endemic countries. Therefore, diagnosis of leptospirosis is important for early treatment of infected hosts and a better prognosis and the development of a sensitive confirmatory diagnostic technique for *Leptospira* are required (Hernández-Rodríguez *et al*. 2011). Humans can be infected during occupational and social activities. People who contribute to maintenance and transportation of animals and their products such as meat, milk and hides may be exposed to the disease (Orrego, De León & Rios 2003). This makes leptospirosis a significant concern for human health (Levett 2001). Outbreaks of human leptospirosis are reported from some countries such as India (John 1996), Japan (Nakamura *et al*. 2006) and Brazil (McBride *et al.* 2005). Leptospires infect the proximal renal tubules of various mammals and are excreted in the urine. Cattle are maintenance hosts for some serovars of leptospirosis and are of importance in transmission of the infection to humans because they excrete live *Leptospira* spp. in their urine for prolonged periods (Levett 2001). In the abattoir, the macroscopic lesions of bovine leptospirosis consist of multifocal white spots in the renal parenchyma. These lesions reflect non-suppurative multifocal interstitial nephritis, which is commonly found in the kidneys of cows infected by leptospires (Wang *et al.* 1999; Yang *et al.* 2001) but they are not pathognomonic for leptospirosis because the same lesion occurs in the kidneys of cows infected by septicaemic colibacillosis (Barker, Van Dreumel & Palmer 1993), salmonellosis or brucellosis (Maxie 1993) and malignant catarrhal fever (McGavin & Zachary 2007). Affected kidneys are not suitable for human consumption and are condemned during meat inspection (Anon 1997).

In the present study, a PCR technique targeting *LipL32* was used for detection of *Leptospira* in bovine kidney, urine and blood samples. Polymerase chain reaction is a rapid, sensitive, inexpensive assay and can identify low doses of bacteria (Céspedes *et al*. 2007). This technique is useful for diagnosis of fastidious and slow-growing organisms, and can be used easily even in non-specialised laboratories compared with an older test, MAT, which is expensive and needs a special laboratory (Céspedes *et al*.2007; Hernández-Rodríguez *et al.* 2011). Procedures based on nucleic acid detection appear to be reliable for differentiation of pathogenic from nonpathogenic *Leptospira* species (Brenner *et al*. 1999; Maxie 1993). Hernández-Rodríguez *et al*. (2011) investigated bovine leptospirosis with PCR, culture and dark field microscopy and reported that PCR is a reliable, rapid and accurate technique for diagnosis of leptospirosis.

The primer used in the current study was based on the *LipL32* gene. This gene is a major outer membrane protein that is found on the surface of all pathogenic *Leptospira* spp. and has been highly conserved amongst these species (Guerreiro *et al*. 2001; Haake *et al*. 2000; Stoddard *et al*. 2009). The amplification of the *LipL32* gene has proved to be a valuable tool for identifying pathogenic leptospires in water samples (Vital-Brazil *et al.* 2010). Cheema *et al*. (2007) used PCR based on *LipL21* and *LipL32* genes for detection of pathogenic *Leptospira* in the serum and tissue samples of cattle and buffaloes and found that both genes are useful for diagnosis of leptospirosis. Jouglard *et al*. (2006) evaluated a PCR method for detection of *Leptospira* spp. Applied primers were designed to amplify a 264 bp region within the *LipL32* gene that is absent in nonpathogenic *Leptospira* species. The sensitivity and specificity were assessed using 7 and 37 saprophytic and pathogenic serovars respectively and 15 other microorganisms. They described this method as very specific for identification of pathogenic serovars.

In this study, the level of detection of leptospiral DNA in kidneys with interstitial nephritis suggests that *Leptospira* spp. are associated with white-spotted kidneys in this area. In a similar study, Mineiro *et al*. (2011) investigated possible leptospiral infection in the sera of 60 slaughtered cows by MAT, and 23 (38.3%) positive samples were obtained. The kidneys of 20 serologically positive cows were examined histopathologically. All of them (100%) showed multifocal interstitial nephritis (Mineiro *et al.* 2011). Yener and Keles

(2001) studied 68 white-spotted and 30 apparently normal kidneys in slaughtered cows for detection of *Leptospira* using histopathology and immunohistochemistry. *Leptospira interrogans* antigen was found in 21 of 68 white-spotted kidneys and 4 of 30 grossly normal kidneys. Grégoire, Higgins and Robinson (1987) studied 955 beef cattle at an abattoir in Quebec, of which 122 kidneys (13%) revealed focal interstitial nephritis. Nephritic kidneys were cultured for leptospirosis, and matching blood samples were examined serologically. Leptospires were isolated from 35 (29%) of the kidneys, and 29 (24%) and 13 (10%) cattle were serologically positive for antibodies to *L. interrogans* serovars *hardjo* and *pomona* respectively. Dorjee *et al*. (2009) found a significant association between white-spotted kidneys and sheep that were serologically positive for *Leptospira* by MAT.

In contrast to the present study, Uzal *et al.* (2002) evaluated the correlation between white-spotted kidneys and leptospirosis and other infective pathogens in slaughtered cows in Australia by MAT and culture methods. They isolated *Leptospira borgpeterseni hardjo* from a urine sample in an adult cow, urine and kidney of another cow and 6 serum samples of cows with white spots in their kidneys, and concluded that *Leptospira* spp. are not associated with white-spotted kidneys. Skilbeck, Forsyth and Dohnt (1988) isolated *L. interrogans* serovar *hardjo* from 18 (8.3%) out of 218 bovine kidneys in Australia. Histopathological lesions related to leptospirosis were not observed in any of the infected kidneys. Leptospires were identified by immunogold silver staining in only two of the kidneys (Skilbeck *et al.* 1988).

In this study, 29.2% of cows with lesions in their kidneys were serologically positive by PCR. Polymerase chain reaction can be diagnostic in the leptospiraemic phase before antibodies are detectible in the serum. When antibodies appear in the blood, leptospires are eliminated from the serum and localised in the kidneys. The organisms have a tendency to localise in kidneys when the acute phase comes to an end (Oliveira *et al*. 2005). In this study, some urine samples of cows with positive kidneys were negative by PCR because the excretion of leptospires in the urine may be intermittent or continuous (Faine *et al*. 1999). Demonstration of leptospires in the kidney or urine when obvious clinical signs are not observed is diagnostic for chronic infection (Ellis 1999).

Since *Leptospira* bacteria can be present in a wide variety of environments, a rapid and specific diagnostic technique is essential for detecting them and distinguishing pathogenic from nonpathogenic *Leptospira* species. This will greatly assist in the application of suitable prevention and control strategies and improvement of epidemiological studies to protect humans, especially those at the risk of infection. The present data indicate that *LipL32*-based primers are useful for diagnosis of leptospirosis and that white-spotted kidneys can be due to leptospirosis in this region of Iran. This is of importance in public health and indicates a significantly increased risk of zoonotic disease, therefore veterinarians and abattoir workers in charge of meat processing should be aware of the potential risks associated with leptospirosis (Orrego *et al.* 2003).

Limitations of the study

In this study, there were limitations that prevented identification of the serovars that resulted in positive samples, but in another study in this geographic area Jafari Dehkordi, Shahbazkia and Ronagh (2011) identified pathogenic serovars of *L. interrogans* in 200 (100 urine and 100 blood) samples of dairy cattle by PCR. Their results showed that 28% of urine and 23% of plasma samples were positive. The main serovars identified were *icterohaemorrhagiae* (50%) and *pomona* (37.5%). The urine samples of 17 serologically negative cows were positive for *Leptospira*. They proposed that these dairy cows were reservoirs and could transmit infection to humans (Jafari Dehkordi *et al.* 2011). Ebrahimi, Nasr and Kojouri (2004) identified *grippotyphosa* (21.33%), *hardjo* (17.33%), *icterohaemorrhagiae* (6.66%) and *pomona* (4%) from 400 serum samples of dairy cattle by MAT.

Recommendations

The results of this study can be used to provide recommendations for veterinarians and workers in slaughterhouses to be more aware of the transmission of leptospirosis and its risks for humans. More research is needed to identify different leptospiral serovars in cows and other ruminants. Also, it is necessary for different molecular and other techniques to be compared to find a reliable, inexpensive and accessible method for serovar identification.

Conclusion

From this investigation, it can be concluded that economic losses due to leptospirosis, including marketing and export restrictions, decrease in weight of infected animals, stillbirths, infertility, abortion and loss of milk production due to mastitis are considerable in this geographic situation and should be given more attention. Although leptospirosis is more prevalent in tropical and subtropical areas (Ramadass & Marshall 1990), this study shows that it can also occur in highland areas with a cold climate.

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Competing interests

The authors declare that they have no conflict of interest and financial disclosure to anybody or any organisations.

Authors' contributions

S.A. (Islamic Azad University) was the project leader and designer, and pathologist, E.T. (Islamic Azad University) and for molecular technique, M.R.H. (Islamic Azad University), M.G.V. (Islamic Azad University) and H.S. (Islamic Azad University) were responsible for collecting the samples and doing some experiments, and also, A.O. (Shiraz University) did pathologic studies.

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